Recombination of strain O segments to HCpro-encoding sequence of strain N of *Potato virus Y* modulates necrosis induced in tobacco and in potatoes carrying resistance genes *Ny* or *Nc*

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SUMMARY

Hypersensitive resistance (HR) to strains O and C of *Potato virus Y* (PVY, genus *Potyvirus*) is conferred by potato genes *Ny_{tr}* and *Nc_{tr}* respectively; however, PVY N strains overcome these resistance genes. The viral helper component proteinases (HCpro, 456 amino acids) from PVY^N and PVY^O are distinguished by an eight-amino-acid signature sequence, causing HCpro to fold into alternative conformations. Substitution of only two residues (K269R and R270K) of the eight-amino-acid signature in PVY^N HCpro was needed to convert the three-dimensional (3D) model of PVY^N HCpro to a PVY^O-like conformation and render PVY^N avirulent in the presence of *Ny_{tr}*, whereas four amino acid substitutions were necessary to change PVY^O HCpro to a PVY^N-like conformation. Hence, the HCpro conformation rather than other features ascribed to the sequence were essential for recognition by *Ny_{tr}*. The 3D model of PVY^C HCpro closely resembled PVY^O, but differed from PVY^N HCpro. HCpro of all strains was structurally similar to β -catenin. Sixteen PVY^N605-based chimeras were inoculated to potato cv. Pentland Crown (*Ny_{tr}*), King Edward (*Nc_{tr}*) and Pentland Ivory (*Ny_{tr}/Nc_{tr}*). Eleven chimeras induced necrotic local lesions and caused no systemic infection, and thus differed from both parental viruses that infected King Edward systemically, and from PVY^N605 that infected Pentland Crown and Pentland Ivory systemically. These 11 chimeras triggered both *Ny_{tr}* and *Nc_{tr}* and, in addition, six induced veinal necrosis in tobacco. Further, specific amino acid residues were found to have an additive impact on necrosis. These results shed new light on the causes of PVY-related necrotic symptoms in potato.

Keywords: hypersensitive resistance, *Potato virus Y*, recombination, resistance gene, tobacco veinal necrosis, tuber necrosis symptoms.

INTRODUCTION

Potyviruses (genus *Potyvirus*) comprise the largest group of positive-sense, single-stranded RNA viruses infecting plants (Adams *et al.*, 2012). *Potato virus Y* (PVY) is the type member of the genus *Potyvirus* and is economically the most important virus infecting potatoes (*Solanum tuberosum* L.) and many other crops of the family Solanaceae (Valkonen, 2007). Losses in potato production caused by PVY can be controlled by growing resistant cultivars. The dominant resistance genes *Nc_{tr}*, *Ny_{tr}* and *Nz_{tr}* found in many potato cultivars each recognize a subgroup of PVY strains on a gene-for-gene basis, triggering a hypersensitive resistance (HR) response typically observed as the development of necrotic local lesions (NLLs) at the initial infection sites (Celebi-Toprak *et al.*, 2002; Chikh-Ali *et al.*, 2014; Cockerham, 1970; Jones, 1990; Moury *et al.*, 2011; Ross, 1986; Rowley *et al.*, 2015). HR restricts the movement of the virus from the initial infection sites, thereby preventing systemic infection, but necrosis *per se* is not sufficient or essential for the restriction of viral movement. Other host factors involved in the recognition of the avirulence protein or signalling for HR, as well as light intensity and/or quality, influence the outcome of HR (Caplan *et al.*, 2008; Jin *et al.*, 2003; Komatsu *et al.*, 2010; Valkonen *et al.*, 1998). Hence, depending on the potato cultivar and environmental conditions, necrotic responses may vary phenotypically and, in addition to NLL formation, PVY may spread systemically causing leaf and top necrosis in the plant (Ross, 1986; Valkonen *et al.*, 1998).

To standardize the results of different laboratories and to avoid variation of results caused by the aforementioned factors, it is recommended to designate PVY isolates to strain groups using standard indicator potato cultivars (Singh *et al.*, 2008). PVY strains triggering HR in the presence of *Nc_{tr}* (cv. King Edward, *Nc:ny:nz*) belong to the strain group PVY^C, whereas those triggering *Ny_{tr}* in cvs. Pentland Crown and Desiree (*nc:Ny:nz*) belong to PVY^O. The PVY strains causing HR in the presence of *Nz_{tr}* (cvs. Pentland Ivory and Maris Bard, *Nc:Ny:Nz*), but not in the other aforementioned cultivars, belong to PVY^Z (Jones, 1990; Kerlan *et al.*, 2011; Singh *et al.*, 2008). This PVY strain group concept serves for the purposes of resistance breeding in potato and is meant to be used with PVY isolates from potato (Cockerham, 1970; Ross, 1986; Singh *et al.*, 2008; Valkonen *et al.*, 1994). In

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this context, it is important to note that many PVY^C strains are not adapted to potato. Phylogenetic grouping based on the viral coat protein (CP)-encoding sequences reveals two clades of PVY^C: clade 2 contains the majority of PVY^C strains isolated from and adapted to potato, whereas clade 1 includes mostly PVY strains that infect other hosts, such as pepper (*Capsicum* spp.) or tobacco (*Nicotiana tabacum* L.), and may show poor infectivity on potato (Blanco-Urgoiti *et al.*, 1998; Moury, 2010). Testing non-potato-adapted PVY isolates on the indicator potato cultivars may produce inconsistent results, as found with the pepper isolate SON41p tested on cv. King Edward (Moury, 2010; Moury *et al.*, 2011). Responses to PVY observed in tobacco plants are used as a supplementary trait in the characterization of PVY strains from potato. The PVY strains overcoming *Ny*, *Nc* and *Nz* and causing veinal necrosis in tobacco plants are designated to the PVY^N strain group, whereas the strains overcoming the three resistance genes, but causing no veinal necrosis in tobacco, are placed in the strain group PVY^E (Galvino-Costa *et al.*, 2012; Singh *et al.*, 2008).

PVY^N and PVY^O can co-infect plants in the field (Bawden and Kassanis, 1951), introducing a risk for recombination of the strains. Indeed, many recombinants of PVY^N and PVY^O differing in the number and positions of recombination sites in the genome have been described in the last two decades (e.g. Chikh-Ali *et al.*, 2008; Glais *et al.*, 2002; Karasev *et al.*, 2011; Kerlan *et al.*, 2011; reviewed by Karasev and Gray, 2013 and Singh *et al.*, 2008). Many overcome *Nc_{tr}* and *Ny_{tr}*. Some cause veinal necrosis in tobacco, but, unlike 'traditional' PVY^N and PVY^O, also induce necrotic symptoms in potato tubers. Others cause necrosis in potato tubers, but no veinal necrosis in tobacco. A few PVY recombinants seem to be recognized and controlled by *Nz* (Kerlan *et al.*, 2011; Quintero-Ferrer *et al.*, 2014). The occurrence of diverse recombinant strains of PVY stresses the importance of their classification and naming according to the PVY strain group concept, emphasizing recognition by the PVY-specific HR genes of potato (Singh *et al.*, 2008). Such classification indicates whether or not the strain can be controlled with the resistance genes available in potato cultivars (Rowley *et al.*, 2015). In contrast, prediction of the phenotypic properties based on the genetic architecture of recombinants is not reliable, unless the causality between the genetic features of the virus and infection phenotypes in potato plants is experimentally established in studies using mutagenized infectious clones of PVY.

The avirulence determinants recognized by *Nc_{tr}* (King Edward) and *Ny_{tr}* (Pentland Crown) have been mapped to the helper component proteinase (HCpro, 456 residues) in PVY^C-SON41p (Moury *et al.*, 2011) and PVY^O-UK (Tian and Valkonen, 2013), respectively. In PVY^O, eight amino acids within a region defined by residues 236–301 in the central part of HCpro determine recognition by *Ny_{tr}*. The eight-amino-acid signature distinguishing PVY^O from PVY^N (I₂₃₆N/T, K₂₃₈L, S₂₄₇A, V₂₅₂I, Q₂₆₂R, R₂₆₉K, K₂₇₀R and

I₃₀₁V, respectively) causes HCpro to fold into alternative conformations and is conserved in the two strain groups (Tian and Valkonen, 2013). The determinants in HCpro of PVY^C triggering *Nc_{tr}* remain to be identified but, in the HCpro of PVY^C-SON41p, the residues 326–355 in the C-proximal proteinase region induce *Nc_{sp}* in the wild potato species *Solanum sparsipilum* (Bitter) Juz. & Bukasov (Moury *et al.*, 2011). [The numbering used here has been adapted from Adams *et al.*, (2005) following the newly determined position of the P1/HCpro cleavage site of PVY, which is nine residues downstream from the original position used for the numbering of residues by Tribodet *et al.* (2005) and Moury *et al.* (2011)]. The avirulence determinants corresponding to *Nz_{tr}* remain to be identified. In PVY^N, HCpro contains determinants of tobacco veinal necrosis symptoms, including residues N₃₃₀, K₃₉₁ and E₄₁₀ (Faurez *et al.*, 2012; Tribodet *et al.*, 2005). Additional determinants are found in the viral polyprotein region spanning from the C-terminus of the cylindrical inclusion protein (CI) to the proteinase domain of the nuclear inclusion protein a (NIa) (Faurez *et al.*, 2012).

Most HCpro functions are controlled by the central region of the protein containing the following conserved amino acid motifs (amino acid residues numbered according to PVY^N605, GenBank accession no. X97895): (i) the FRNK box involved in non-specific RNA binding, virus movement and viral amplification; (ii) the RNA binding motifs RNP-2 (amino acids 246–251) and RNP-1 (amino acids 282–289); (iii) the CCCT motif (amino acids 290–293) associated with long-distance viral movement in host plants; and (iv) a hypervariable region (HVR) (amino acids 328–333) which may indirectly control host defence signalling by affecting the HCpro conformation (Haikonen *et al.*, 2013b). A translation initiation factor 4E binding domain (amino acids 343–349) is situated at the border of the central and proteinase domains (Ala-Poikela *et al.*, 2011). HCpro also interacts with many other host proteins (Ala-Poikela *et al.*, 2011; Haikonen *et al.*, 2013b) and with viral proteins, most notably CP (Seo *et al.*, 2010).

The structure of the C-proximal proteinase domain of HCpro has been resolved (Guo *et al.*, 2011). Furthermore, three-dimensional (3D) modelling of HCpro has predicted major differences in the central portions of HCpro in PVY^O and PVY^N, suggesting that a change in HCpro conformation may be required for the specific recognition of PVY^O by *Ny_{tr}* (Tian and Valkonen, 2013). The aim of this study was to further elucidate the molecular determinants involved in the recognition of PVY^O by *Ny_{tr}*. Furthermore, because the necrotic diseases caused by recombinant PVY strains in potato have become a widespread problem (Karasev and Gray, 2013), we aimed to elucidate the genetic determinants of HCpro causing the induction of necrotic symptoms in potato by PVY^N recombinants that carry genomic segments of PVY^O and infect potato cultivars carrying *Ny_{tr}* and/or *Nc_{tr}*. The 3D conformation of HCpro mutants was predicted with the aim to understand possible structure–function relationships.

RESULTS

Impact of PVY HCpro mutations in potato cvs. Pentland Crown and Pentland Ivory

Previously, 3D conformational modelling has predicted that amino acid substitutions K269R and R270K in the HCpro of PVY^N605 significantly alter the 3D structure of the protein's central region, making its conformation similar to that of HCpro of PVY^OUK (Tian and Valkonen, 2013). Here, substitutions K269R and R270K were

introduced to the infectious clone of PVY^N605. The resulting mutant PVY^N605-RK (Fig. 1) was propagated in tobacco plants (*N. tabacum* cv. Samsun nn), and sap from systemically infected leaves was used to inoculate full-grown leaves of the potato cultivars Pentland Crown (*Ny.nc*) and Pentland Ivory (*Ny.Nc*). PVY^N605-RK caused veinal necrosis in systemically infected tobacco leaves (Table 1) and NLLs in inoculated leaves of both potato cultivars (Fig. 2). These results are consistent with the model in which *Ny_{ibr}* recognizes the 3D conformation of PVY^O HCpro. They further indicate that the avirulence determinant that

Fig. 1 Genomic organization in wild-type *Potato virus Y* (PVY), viral mutants and chimeras. The proteolytic cleavage sites for mature proteins are indicated by vertical lines for PVY^OUK (top white bar) and the enlarged N-proximal part of PVY^N605 (black bar, construct 1). The position of the ribosomal frameshifting nucleotide (nucleotide 2919 in PVY^N605), resulting in an additional short open reading frame (PIPO) embedded within the P3 cistron and expressed as a P3-PIPO fusion product in potyviruses (Adams *et al.*, 2012), is indicated by a vertical dotted line. 5'-UTR and 3'-UTR refer to untranslated regions at the termini of the genome. The infectious clone PVY^N605 was modified by replacing the entire 5'-terminus (2592 nucleotides; chimera 5) or shorter regions (chimeras 6–17) with the corresponding regions of PVY^OUK. The recombination points and corresponding regions of the genome are indicated with reference to the polyprotein amino acid positions at the bottom of the figure. Site-directed mutagenesis was also carried out. Mutated positions are indicated with white (chimeras 2–4 and 8) or black (chimeras 10–16) vertical dotted lines, and mutated residues in specific regions (α and β in HCpro; γ and δ in P3) are specified under construct 18. In construct 2 (NP3mut), three residues in P3 of PVY^N605 were converted to PVY^OUK-like, whereas, in construct 18 (N'), the PVY^OUK-derived region in construct 6 (O5) was replaced with the original PVY^N605 sequence. Columns on the right indicate whether the virus elicited a hypersensitive resistance response and necrotic local lesions (NLLs) in potato cultivars Pentland Crown and Pentland Ivory carrying *Ny_{ibr}*, necrotic lesions (NLs) in potato cv. King Edward (K.E.) or veinal necrosis (VN) in systemically infected tobacco leaves (N.t.). The two plus signs that are in parentheses in the middle column indicate that only a few necrotic lesions were observed.

Table 1 Symptoms in inoculated leaves of potato cultivars Pentland Crown, Pentland Ivory and King Edward at 11 days post-inoculation (dpi) and in the upper non-inoculated leaves of King Edward and tobacco cultivar Samsun nn at 14 dpi.

	Pentland Crown (<i>Ny:nc</i>)	Pentland Ivory (<i>Ny:Nc</i>)	King Edward (<i>ny:Nc</i>)		Tobacco (<i>Nicotiana tabacum</i> cv. Samsun nn)
			Inoculated leaves	Upper non-inoculated leaves	
[Control or mock]	ns, nvd	ns, nvd	ns, nvd	ns, nvd	ns, nvd
PVY ⁰ UK	NLL	NLL	BS/GS	M, VN	M
PVY ^N 605	ns, v +	ns, v +	ns, v +	ns, v +	VN
NP3mut	ns, v +	ns, v +	ns, v +	ns, v +	VN
PVY ^N 605-F	ns, v +	ns, v +	ns, v +	ns, v +	VN
PVY ^N 605-RK	NLL	NLL	NLL	ns, nvd	VN
5'UTRO5	NLL	NLL	NLL	ns, nvd	M
O5	NLL	NLL	NLL	ns, nvd	M
O5ON	ns, nvd	ns, nvd	ns, v +	ns, v +	VN
O5ON-F	ns, v +	ns, v +	ns, v +	ns, v +	VN
O5NO	NLL	NLL	ns/NLL (v+)*	ns, nvd	M
O5NO-4-47	NLL	NLL	ns/NLL (v+)*	ns, nvd	M
O5NO-2-22	NLL	NLL	NLL	ns, nvd	M
O5NO-6-13	NLL	NLL	NLL	ns, nvd	VN
O5NO-6-13N	NLL	NLL	NLL	ns, nvd	VN
O5NO-6-13N-Y	NLL	NLL	NLL	ns, nvd	VN
O5NO-6-13N-KR	NLL	NLL	NLL	ns, nvd	VN
O5NO-6-13N-RKR	NLL	NLL	NLL	ns, nvd	VN
O5NNO45	ns, v +	ns, v +	ns, v +	ns, v +	M
N'	ns, v +	ns, v +	ns, v +	ns, v +	VN

*Potato virus Y (PVY) detected by enzyme-linked immunosorbent assay (ELISA) in some, but not all, inoculated leaves.

BS/GS, brown/greenish lesions; M, mosaic symptoms; NLL, necrotic local lesions and no systemic infection; ns, no symptoms; v +, virus detected by ELISA; nvd, no virus detected by ELISA; VN, veinal necrosis.

triggers *Ny_{thr}* is different from the determinant responsible for PVY^N-induced veinal necrosis in tobacco.

The chimera O5NO-6-13N (Fig. 1) differs from PVY^N605 by only nine amino acid residues within the amino acid region 227–327, which is identical to PVY⁰UK (Tian and Valkonen, 2013). Eight of the nine residues, including R₂₆₉ and K₂₇₀, constitute the PVY⁰ amino acid signature distinguishing PVY⁰ and PVY^N strains. The ninth residue, F₃₂₃, is found in all PVY⁰ strains and a few PVY^N strains (Tian and Valkonen, 2013). Substitutions R269K and K270R (chimera O5NO-6-13N-KR) and an additional substitution (Q262R; chimera O5NO-6-13N-RKR) were introduced to O5NO-6-13N (Fig. 1). The resulting two chimeras were propagated in tobacco and sap inoculated to Pentland Crown and Pentland Ivory, as described above. Both chimeras caused NLLs in inoculated leaves of both potato cultivars, indicating that the introduced N-like amino acid residues did not affect recognition by *Ny_{thr}*. The 3D conformation of HCpro was similar in PVY⁰UK, O5NO-6-13N, O5NO-6-13N-KR and O5NO-6-13N-RKR (Fig. 2), as modelled by I-TASSER using previously described procedures (Tian and Valkonen, 2013). Taken together, these results were consistent with the hypothesized 3D conformation-based recognition of PVY⁰ HCpro by *Ny_{thr}*.

In addition, we modelled the structure of a mutant based on O5NO-6-13N, in which all the last four residues of the PVY⁰ amino acid signature were substituted for the corresponding residues of PVY^N605 (O5NO-6-13N-RKRV). This protein was predicted to

take on the 3D conformation of PVY^N605, in contrast with the previously modelled O5NO-6-13N-based construct, in which substitution of the first four amino acid residues of the PVY⁰ amino acid signature for the corresponding residues of PVY^N605 (O5NO-6-13N-NLAI) gave a predicted 3D conformation similar to that of PVY⁰UK (Fig. S1, see Supporting Information).

Mutations in PVY HCpro alter responses in potato cv. King Edward

A series of viral mutants and chimeras was prepared by: (i) substituting amino acid residues in PVY^N605 for the corresponding residues of PVY⁰UK; (ii) replacing genomic segments of PVY^N605 with the corresponding PVY⁰UK segments; or (iii) substituting amino acid residues in PVY⁰UK-derived genomic segments in the chimera for the corresponding residues of PVY^N605 (Fig. 1). As PVY⁰UK and PVY^N605 both infect King Edward (*ny:Nc*) systemically, all mutants and chimeras were expected to do the same. Although all 16 mutants and chimeras infected tobacco plants systemically, only five were found to cause systemic infection in King Edward (Table 1, Fig. 1). These included NP3mut (construct 2, with three O-like amino acid substitutions in the N-terminus of P3), O5ON (construct 7, with the N-proximal part of HCpro and most of the P1 region substituted for the corresponding segment of PVY⁰UK), PVY^N605-F and O5ON-F (constructs 3 and 8, both with F₃₂₃ substituted for Y₃₂₃ in HCpro) and O5NNO45 [construct 17,

Fig. 3 Induction of necrotic lesions in inoculated leaves of potato cv. King Edward at 12 days post-inoculation with PVY^N605, PVY⁰UK and various mutants and chimeras (see Fig. 1). The apical leaflet (c. 5–7 cm in length) of the inoculated leaf is shown. PVY, *Potato virus Y*.

with a PVY⁰UK-derived genomic segment encoding the C-proximal part of HCpro and the N-terminus of P3 (residues 631–802, including the HCpro/P3 proteolytic cleavage site) substituted for the corresponding segment in PVY^N605]. All 11 PVY mutants and chimeras that failed to infect King Edward systemically (constructs 4–6 and 9–16) also failed to infect Pentland Crown and Pentland Ivory systemically and induced HR and NLLs in these two cultivars (Figs 1 and 2). Nine of these induced necrotic lesions also in inoculated leaves of King Edward. The lesions caused by many chimeras (e.g. O5NO-6-13-Y) in King Edward were similar to the characteristic HR-associated NLLs observed in Pentland Crown and Pentland Ivory, although they

were more variable in number and size in King Edward than in Pentland Crown and Pentland Ivory (Figs 2 and 3).

The 5'-proximal portion of the PVY^N605 genome was replaced entirely (chimera 5'UTRO5) or in its majority (chimera O5) with the corresponding genomic segment of PVY⁰UK, up to the N-proximal part of the P3-encoding region (constructs 5 and 6, Fig. 1). These chimeras caused larger NLLs in King Edward than any other virus tested (Fig. 3), but failed to infect King Edward systemically (Table 1). The multiplication efficiency of 5'UTRO5 and O5 in tobacco leaves is exceptionally high, reaching over 600 µg PVY CP antigen/g leaf tissue, with only chimera O5NNO45 reaching similarly elevated levels (Fig. 1); PVY⁰UK, PVY^N605 and most PVY

chimeras accumulated <200 µg PVY CP antigen/g leaf tissue (Tian and Valkonen, 2013). The high multiplication efficiency might have contributed to efficient cell-to-cell movement and to the development of larger lesions in leaves inoculated with 5'UTRO5 and O5. However, this is not the reason for the induction of necrotic lesions by 5'UTRO5 and O5, because O5NNO45, which accumulated at similarly high titres, caused no symptoms in inoculated leaves and infected King Edward systemically and asymptotically. No veinal necrosis was observed in tobacco plants systemically infected with PVY^OUK, 5'UTRO5 or O5 (Table 1).

Chimera O5NO (construct 9) contains the C-proximal part of HCpro and the N-terminus of P3 derived from PVY^OUK, whereas, in O5NO-4-47 (construct 10), amino acid substitutions were performed to introduce four PVY^N605 residues into the PVY^OUK-derived segment of HCpro (D330N, K338R, Y341F and V346I) (Fig. 1). Both constructs induced few, very small, brown or black lesions or no lesions at all (Fig. 3). Two amino acid substitutions (R391K and D410E) introduced into O5NO in the PVY^O-derived segment (chimera O5NO-2-22) consistently caused necrotic lesions in inoculated leaves of King Edward, as did all other O5NO-based viral mutants carrying residues K₃₉₁ and E₄₁₀ (Figs 1 and 3). However, chimera O5NNO45, which carries a shorter segment derived from PVY^OUK (amino acids 347–456; Fig. 1), caused no symptoms in inoculated and systemically infected leaves of King Edward (Fig. 3). Hence, HCpro residues K₃₉₁ and E₄₁₀ were essential for the induction of necrotic lesions in leaves of King Edward (Fig. 1).

Taken together, 11 of the 16 chimeras and mutants tested exhibited novel infection phenotypes. PVY^OUK induced green-brownish lesions in inoculated leaves of King Edward (Fig. 3), but the lesions developed late [10–15 days post-inoculation (dpi)] and were phenotypically different from NLLs caused by PVY^O in Pentland Ivory and Pentland Crown (Fig. 2). They were not associated with HR, because PVY^OUK infected King Edward systemically and caused veinal necrosis in systemically infected leaves at 21 dpi (Table 1). PVY^N605 caused no symptoms in inoculated leaves of King Edward (Fig. 3), and mild mosaic symptoms were observed in systemically infected leaves (Table 1). Therefore, the 11 chimeras which caused local lesions in King Edward and failed to infect this cultivar systemically differed from both parental viruses. Furthermore, the large NLLs caused by 5'UTRO5 and O5 differed from the lesions induced by any other virus strain or chimera tested. Previous studies have shown that a chimera of PVY^N605 encoding the HCpro of PVY^C triggers *Nc_{trr}*, HR and NLLs in King Edward (Moury *et al.*, 2011). However, our results show that 5'UTRO5 and O5, which contain the complete HCpro-encoding sequence of PVY^OUK, also elicited an HR-like response and the development of NLLs in King Edward. Indeed, all PVY^N chimeras containing a PVY^O-like C-proximal part of HCpro induced at least some NLLs on King Edward (Fig. 1).

Combination of HCpro residues needed for the induction of veinal necrosis in tobacco

Chimera O5NO-2-22 contains the residues K₃₉₁ and E₄₁₀ needed for the induction of necrotic lesions in leaves of King Edward, but did not cause veinal necrosis in tobacco. Instead, mosaic symptoms and vein clearing were observed in systemically infected tobacco leaves (Table 1). When four additional amino acid substitutions (D330N, K338R, Y341F and V346I; mutated region α in Fig. 1) were introduced into O5NO-2-22 to make the PVY^O-derived HCpro segment more PVY^N-like (chimera O5NO-6-13), veinal necrosis was induced in systemically infected tobacco leaves. Similar results were obtained with O5NO-6-13-based mutants and other chimeras carrying these six PVY^N-like amino acid residues (Fig. 1). In contrast, chimera O5NNO45 caused mosaic symptoms in systemically infected tobacco leaves, which confirmed that residues N₃₃₀, R₃₃₈, F₃₄₁ and I₃₄₆ in HCpro are not sufficient to induce veinal necrosis in tobacco, as K₃₉₁ and E₄₁₀ are also required (Fig. 1).

Residue Phe₃₂₃ in HCpro influences PVY accumulation

The role of residue 323 was tested by substituting F₃₂₃ for Y in chimera O5NO-6-13N (mutant O5NO-6-13N-Y), and by substituting Y₃₂₃ for F in both PVY^N605 (mutant PVY^N605-F) and chimera O5ON (mutant O5ON-F) (Fig. 1). These substitutions did not affect symptom expression in potato cultivars carrying *Ny_{trr}*, because PVY^N605, PVY^N605-F, O5ON and O5ON-F caused no lesions on leaves of Pentland Crown and Pentland Ivory (Fig. 1), whereas O5NO-6-13N and O5NO-6-13N-Y both induced NLLs in both cultivars (Fig. 2).

Virus accumulation in inoculated leaves was estimated by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using non-PVY-strain-specific polyclonal anti-CP antibodies, with known amounts of purified PVY particles for comparison, as described by Tian and Valkonen (2013). The amounts of PVY CP antigen in inoculated potato leaves were approximately five-fold higher with PVY^N605-F (79.8 ± 61.7 µg/g leaf in Pentland Crown, 65.2 ± 42.3 µg/g in Pentland Ivory and 46.0 ± 28.0 µg/g in King Edward; $n = 3$, $P < 0.05$) than with PVY^N605 (12.5 ± 17.4, 12.6 ± 4.2 and 7.5 ± 2.6 µg/g, respectively). The accumulation of O5ON was very low in inoculated leaves of Pentland Crown (0.04 ± 0.04 µg/g leaf), and below the detection threshold (0.64 ng/g leaf) in inoculated leaves of Pentland Ivory and King Edward. In contrast, the accumulation of O5ON-F was readily detected in inoculated leaves of all three cultivars (1.4 ± 0.4, 1.0 ± 1.1 and 0.8 ± 0.6 µg/g in Pentland Crown, Pentland Ivory and King Edward, respectively).

Substituting F₃₂₃ for Y in chimeras containing the PVY^O amino acid signature had little effect on virus accumulation in inoculated leaves. The amounts of CP antigen with O5NO-6-13N and O5NO-6-13N-Y (Fig. 1) were similar in Pentland Crown (0.4 ± 0.2 and 0.3

$\pm 0.4 \mu\text{g/g}$, respectively), Pentland Ivory (1.0 ± 0.3 and $2.2 \pm 1.9 \mu\text{g/g}$, respectively) and King Edward (0.8 ± 0.3 and $1.5 \pm 0.6 \mu\text{g/g}$, respectively).

Modelling with I-TASSER predicted that the 3D conformation of O5NO-6-13N-Y would be similar to that of O5NO-6-13N. However, substituting Y₃₂₃ for F in PVY^N605 (mutant PVY^N605-F) was predicted to alter the protein conformation upstream of the mutation by interrupting an α -helix containing the conserved RNP-1 motif (amino acids 282–289) with a coil structure (Fig. 2).

Predicted conformational differences in HCpro among PVY strain groups

HCpro amino acid sequences of the 265 PVY isolates available in the curated DPVweb database (<http://www.dpvweb.net>) were subjected to phylogenetic analysis, which grouped them tentatively to clusters PVY^N, PVY^O and PVY^C (not all have been classified using the pertinent biological tests). Residues K₂₆₉ and R₂₇₀, whose mutation to R and K, respectively, triggers *Ny_{thr}* and is predicted to convert the protein from an N-like to an O-like conformation, were conserved in the PVY^N cluster (165 isolates). Of the 84 PVY^O isolates, 68 contained the conserved eight-amino-acid PVY^O amino acid signature, and the naturally occurring amino acid substitution in the remaining 16 isolates had no effect on the predicted protein conformation. Of the 16 PVY^C isolates, nine contained the conserved eight-amino-acid PVY^O signature, and the remaining seven differed from the PVY^O amino acid signature at only two or three positions. These data suggest that HCpro shows limited sequence variability and high conformational unity within PVY strain groups.

Pentland Crown (*Ny:nc*) and King Edward (*ny:Nc*) carry a different PVY strain group-specific resistance gene (recognizing PVY^O and PVY^C, respectively). The avirulence determinants of both genes within HCpro are believed to reside at close proximity: amino acids 236–301 for avirulence of *Ny_{thr}* in PVY^O (Tian and Valkonen, 2013), and amino acids 326–356 for avirulence of *Nc_{spl}* (*S. sparsipilum*) in PVY^C (Moury *et al.*, 2011). It was therefore of interest to predict the 3D conformation of HCpro in PVY^C and compare it with both PVY^O and PVY^N. Tertiary structures were modelled with I-TASSER as described by Tian and Valkonen (2013) using HCpro sequences of the PVY^C isolate PRI509 (GenBank accession no. EU563512) (Dulleman *et al.*, 2011) and isolate Adgen (AJ890348) belonging to clade C2 (potato strains), isolate SON41p (AJ439544) belonging to clade 1, and isolate Chile3 (FJ214726) that does not belong to either clade (Moury, 2010). SON41p has been used to localize the avirulence determinant corresponding to *Nc_{spl}* (Moury *et al.*, 2011). Comparison of the 3D models of the four strains with those from PVY^OUK and PVY^N605 suggested that PVY^C and PVY^O are very similar, and are clearly different from PVY^N (amino acids 179–360 shown in Fig. S1). A few differences were detected among the PVY^C strains. In strain SON41, the first two residues of the conserved RNP-1 motif were included in an α -helix and the remaining

residues in a coil structure, which differed from most other PVY^C strains, but was similar to PVY^OUK. In the other three PVY^C strains, the whole RNP-1 motif was coiled. In addition, the CCCT motif was found in an α -helix in the PVY^C strain Chile, but was in a coil structure in the other PVY^C strains and in PVY^OUK (Fig. S1).

I-TASSER provides a list of proteins with the highest structural similarity to the protein under analysis. Modelling the 3D conformation of HCpro in PVY and a few other potyviruses [such as *Potato virus A* (PVA), *Tobacco etch virus* and *Pea seedborne virus*] by I-TASSER indicated that HCpro is most similar to the β -catenins (Fig. S2, see Supporting Information). The β -catenin with highest similarity to PVY HCpro was a cadherin-associated protein of zebrafish (*Danio rerio* F. Hamilton), and the second most similar protein was SYS-1 of a nematode (*Caenorhabditis elegans* Maupas).

Analysis of HCpro in the 265 PVY isolates available in the DPVweb database using ΔG prediction server 1.0 (<http://dgpred.cbr.su.se>; Hessa *et al.*, 2007) predicted a hydrophobic region at amino acids 333–355 in all PVY^N strains, but not in PVY^O strains, and in only a few PVY^C strains. The positive ΔG value (1.303) suggests that HCpro of PVY^N may be a peripherally associated membrane protein, rather than a transmembrane protein.

Analysis by the metaDISORDER webserver (<http://genesilico.pl/metadisorder/>; Kozłowski and Bujnicki, 2012) predicted that a region of PVY HCpro containing a high proportion of basic residues is intrinsically disordered in all PVY strain groups. The length of the predicted disordered region varied among PVY strains and chimeras (Table S1, see Supporting Information), but the residues with the highest probability of belonging to the disordered region were those at positions 269–272 (Fig. 4), which includes the residues whose mutation in PVY^N605 caused a major change in HCpro conformation (Fig. 2).

DISCUSSION

Eight amino acid residues within the central region of HCpro form PVY^O and PVY^N amino acid signatures that clearly and consistently distinguish the PVY^O and PVY^N strains. More importantly, the signatures seem to determine the differing 3D conformations of PVY^O and PVY^N HCpro, which are recognized in PVY^O strains by *Ny_{thr}*, and allow PVY^N to overcome the resistance conferred by this gene. According to structural modelling, only two amino acid substitutions (K269R and R270K) in the PVY^N amino acid signature are required to convert the tertiary structure of PVY^N HCpro to an O-like conformation and trigger *Ny_{thr}* and HR (Tian and Valkonen, 2013). This model is supported by the results of the present study based on the construction and testing of the respective mutant of PVY^N. However, the last four residues in the PVY^O amino acid signature need to be substituted for residues in PVY^N in order to convert the O-like HCpro 3D conformation to an N-like conformation. Therefore, it seems unlikely that PVY^O could overcome *Ny_{thr}* by

Fig. 4 Disordered region predicted in the helper component proteinase (HCpro) of PVY⁰UK using the metaDISORDER webserver. A disorder tendency above 0.5 indicates that the amino acids belong to a disordered region. Residues 264 and 279 define the borders of a predicted disordered region. The region containing the PVY⁰ amino acid signature (amino acids 227–327) is shaded. PVY, *Potato virus Y*.

a series of point mutations in the PVY⁰ amino acid signature. Instead, recombination with a strain belonging to another PVY strain group in a co-infected plant (Bawden and Kassanis, 1951) appears to be a more likely scenario for this conversion, and is supported by the existence of natural recombinants, such as the PVY-Wilga strain (Chrzanowska, 1991; Glais *et al.*, 2002) and its variants in the field. Most of the PVY-Wilga genome is PVY⁰-like, with the exception of HCpro, which is similar to PVY^N (Glais *et al.*, 2002; Hu *et al.*, 2009).

Currently, diverse populations of PVY^N/PVY⁰ recombinants are arising and evolving in potato crops worldwide (e.g. Galvino-Costa *et al.*, 2012; Hu *et al.*, 2009; Karasev *et al.*, 2011; Quintero-Ferrer *et al.*, 2014; Schubert *et al.*, 2007). This study has revealed new details regarding the significant amino acid residues needed in PVY^N/PVY⁰ recombinants to induce necrotic symptoms in potato and tobacco. PVY^N strains induce veinal necrosis in tobacco leaves, for which determinants are found in several regions of the viral polyprotein, including HCpro (Faurez *et al.*, 2012; Tribodet *et al.*, 2005). In our study, the PVY^N605 chimera O5NO, which contains the C-proximal portion of PVY⁰UK HCpro, was helpful in elucidating the induction of necrotic symptoms in potato and tobacco. This chimera induced few if any detectable local lesions in King Edward and no veinal necrosis in tobacco. The introduction of two PVY^N-derived residues, K₃₉₁ and E₄₁₀, to HCpro by point mutations induced a few NLLs in King Edward, but no veinal necrosis in tobacco. The results were similar when four other N-like substitutions (D330N, K338R, Y341F and V346I) were made in the sequence derived from PVY⁰UK. However, when all six amino acid substitutions were combined (chimera O5NO-6-13), necrotic symptoms became more obvious in King Edward and veinal necrosis was seen in tobacco. Other studies have also shown that N₃₃₀, K₃₉₁ and E₄₁₀ are important for the induction of veinal necrosis in tobacco (Faurez *et al.*, 2012; Tribodet *et al.*, 2005), but our results show that specific residues in both closely situated regions of HCpro are required. Further, when additional

N-like residues were introduced to chimera O5NO-6-13 by substituting residues of the PVY⁰ amino acid signature, local lesions became more numerous and less necrotic. These results identify specific amino acid residues that enhance necrotic symptoms in potato (cv. King Edward) and tobacco (cv. Samsun), and underline that amino acids surrounding significant residues can act as a modulator of symptoms. The results also emphasize the significance of the C-proximal part of HCpro in the induction of necrosis in potato and tobacco, which is supported by the results of a previous study on another tobacco cultivar (cv. Xanthi) (Faurez *et al.*, 2012; Tribodet *et al.*, 2005).

The results obtained with strains PVY^N605 and PVY⁰UK were fully consistent with previous studies in which strains of PVY^N and PVY⁰ were shown to overcome resistance controlled by *Nc_{tr}* in King Edward (Jones, 1990). In contrast, the chimeras 5'UTRO5 and O5 of PVY^N, expressing the complete HCpro of PVY⁰, induced obvious and numerous NLLs in King Edward and failed to infect plants systemically, illustrating a clear HR. Indeed, 11 PVY^N/PVY⁰ chimeras in our study were consistently avirulent in the three potato cultivars carrying *Ny_{tr}* (Pentland Crown), *Nc_{tr}* (King Edward) or both (Pentland Ivory). The contribution of *Nz_{tr}* to the necrotic symptoms observed in Pentland Crown remains enigmatic. Its assessment was not possible because of a lack of a control potato variety carrying only *Nz_{tr}*. The results may suggest that *Nc_{tr}* and *Ny_{tr}* recognize similar (structural) determinants in PVY⁰ and PVY^C strains, respectively, which was supported by 3D modelling suggesting that the conformation of HCpro was similar in PVY⁰ and PVY^C; however, these findings do not preclude the existence of minor differences that were not observed in our study. Furthermore, genetic mapping of *Ny_{tr}* and *Nc_{tr}* suggests that they reside in the same gene cluster on potato chromosome IV and may be allelic (Celebi-Toprak *et al.*, 2002; Moury *et al.*, 2011), making it more likely to find them recognizing similar avirulence proteins. However, the specificity in the recognition of wild-type PVY⁰ and PVY^C strains by *Ny_{tr}* and *Nc_{tr}*, respectively, may be explained by

the involvement of other host proteins, as reported for the protein encoded by resistance gene *N* recognizing *Tobacco mosaic virus* in tobacco (Caplan *et al.*, 2008), or a complex of viral and host proteins in recognition. The interactions and complexes may differ between the chimeras and parental virus strains, because the chimeras inducing NLLs in King Edward contained an O-like HCpro conformation, whereas most of the genomic RNA and other proteins produced by these chimeras during infection were those of PVY^N605. The putative additional factors playing a role in specificity will be an interesting topic of future study.

HCpro is a movement protein in potyviruses and, in this study, it was predicted using ΔG prediction server analysis to be membrane associated. This is consistent with similar membrane association reported for movement proteins from other viruses, such as *Prunus necrotic ringspot virus* (Martinez-Gil *et al.*, 2009) and *Tobacco mosaic virus* (Peiró *et al.*, 2014). Furthermore, the ΔG prediction analysis suggested that HCpro of PVY^N contained a transmembrane helix domain formed by residues 333–355, and therefore may be a peripherally associated membrane protein. HCpro of PVY^O did not contain this helix domain and it was rare in PVY^C. The differences in conformation and membrane localization of PVY^N HCpro, compared with PVY^O and PVY^C HCpro, imply differences in interactions with other proteins and cellular receptors. Modelling predicted that when residues 333–355 in PVY^N605 are substituted for the corresponding sequence of PVY^C, the predicted transmembrane helix is lost. In Moury *et al.* (2011), chimera 9 was based on PVY^N605, but part of the HCpro sequence (amino acids 326–355) was replaced with the corresponding sequence from PVY^C SON41p. Chimera 9 induced necrotic lesions in inoculated leaves and apical necrosis in *S. sparsipilum* carrying *Nc_{Spl}*, whereas PVY^N605 caused no symptoms in inoculated leaves and mild mosaic symptoms in systemically infected leaves of *S. sparsipilum*. These results highlight the functional importance of the HCpro transmembrane domain in PVY^N host interactions, and may explain why PVY^N is not recognized by *Ny_{tab}* and *Nc_{tab}*.

Stretches of basic residues in viral proteins often indicate involvement in essential functions (Rajamäki and Valkonen, 2009; Zhang *et al.*, 2013). HCpro modelling predicted a disordered region (amino acids 261–277) containing a stretch of basic residues in the central region of all PVY strains. The disordered region included two amino acid residues (269 and 270) that differ between PVY^O (R₂₆₉K₂₇₀) and PVY^N (K₂₆₉R₂₇₀) strains, and play an important role in protein conformation and recognition by *Ny_{tab}*. The disordered region may allow for multiple molecular interactions, which could be expected for a multifunctional protein. Many functions, including viral movement, genome amplification, RNA binding and RNA silencing suppression, are controlled by the central region of HCpro (Haikonen *et al.*, 2013b; Kekarainen *et al.*, 2002; Seo *et al.*, 2010; reviewed in Gao *et al.*, 2012).

Plant U-box protein family members of E3 ubiquitin ligases are numerous, and most carry tandem armadillo (ARM) repeats that

give rise to conserved protein structures, although the proteins *per se* have little similarity at the amino acid level (Drechsler *et al.*, 2011; Kidd III *et al.*, 2005). An ARM repeat consists of three α -helices and multiple repeats that interact and form interfaces for protein–protein interactions. The best-studied ARM protein is β -catenin, a protein fractal of microtubules that plays a key role in plant cytoskeletal regulation and development (Gardiner *et al.*, 2011). β -Catenins have been implicated in the regulation of pathogen defence and cell death in *Arabidopsis thaliana* (L.) Heynh., rice (*Oryza sativa* L.) and *N. benthamiana* Domin (Mosher *et al.*, 2006; Sharma *et al.*, 2014). They may control some of these functions via interaction with microtubule-binding proteins (Gardiner *et al.*, 2011). The structural similarity between HCpro and β -catenins is intriguing, because HCpro interacts with the microtubule-associated protein HIP2, which controls the accumulation of PVA in infected plants (Haikonen *et al.*, 2013a, b). The interaction with HIP2 is controlled by HCpro amino acids 323–352 in PVY (Haikonen *et al.*, 2013b). Indeed, substitution of the first residue (Y₃₂₃ for F) in the HIP2-binding domain of HCpro in PVY^N605 has a major impact on the HCpro conformation and virus accumulation.

The HIP2-interacting region of HCpro also contains a stretch of six residues (amino acids 328–333 in PVY^N605) that comprise an HVR in potyviruses (Haikonen *et al.*, 2013b). Mutation of the HVR in PVA induces many defence-related genes (including genes involved in ethylene- and jasmonic acid-inducible pathways), induces the onset of necrotic symptoms in systemically infected tobacco leaves and reduces viral fitness (Haikonen *et al.*, 2013b). Mutations in the HVR are predicted to affect the conformation of adjacent HCpro regions, such as those containing RNP-1 and CCCT (Haikonen *et al.*, 2013b). In our study, mutations introduced to the PVY^O/PVY^N amino acid signatures were also predicted to alter the conformation of the aforementioned HCpro region containing conserved motifs. Taken together, these results suggest a scenario in which the amino acid substitutions, which in HCpro overcome PVY strain group-specific resistance genes, alter the HCpro conformation in a manner that may interfere with other host interactions of the multifunctional HCpro and induce other defence- or cell death-related responses. This could explain why many chimeras of PVY^N605 and PVY^OUK displayed infection and symptom phenotypes that were different from either parental virus.

EXPERIMENTAL PROCEDURES

Plants and viruses

Pathogen-indexed potato cvs. Pentland Crown, Pentland Ivory and King Edward were obtained from the *in vitro* collection of the Plant Pathology Laboratory, University of Helsinki, Finland. Plants were grown in a growth room under illumination from sodium halide lamps. The light intensity was 210 $\mu\text{E}/\text{m}^2/\text{s}$ at the plant top level, the photoperiod was 16 h, the relative

humidity was 40% and the temperature was 17–19 °C at night and 19–20 °C during the day. The plants were fertilized weekly with a 1.0% solution of N : P : K = 8:4:6 or 16:9:22 fertilizer (Yara, Espoo, Finland). Potato cultivars were propagated by rooting stem cuttings. *Nicotiana tabacum* Samsun plants were grown from seed.

PVY⁰UK (Valkonen *et al.*, 1991) was propagated in *N. tabacum* Samsun. The complete sequence of PVY⁰UK has been determined [National Center for Biotechnology Information (NCBI) accession no. JX424837]. The infectious clone of the PVY⁶⁰⁵ isolate, driven by the 35S promoter of *Cauliflower mosaic virus*, has been reported (Jakab *et al.*, 1997). A recent version of the clone, PVYN-605(123), stabilized by the introduction of introns (Bukovinski *et al.*, 2007), was kindly provided by E. Maiss, University of Hannover, Germany, and used in this study.

Construction of chimeric viruses

Chimeric clones 2, 5–7, 9–13, 17 and 18 (Fig. 1) have been described previously (Tian and Valkonen, 2013). In constructs 3 and 8 (PVY⁶⁰⁵-F and O5ON-F; Fig. 1), Y₃₂₃ was mutated to F. In construct 14 (O5NO-6-13N-Y), F₃₂₃ was mutated to Y. In construct 4 (PVY⁶⁰⁵-RK), two substitutions (K269R and R270K) were introduced to PVY⁶⁰⁵. Mutations R269K and K270R were also introduced to O5NO-6-13N, resulting in construct 15 (O5NO-6-13N-KR), and an additional mutation (Q262R) was introduced to construct 15 to give construct 16 (O5NO-6-13N-RKR).

Primers for the introduction of mutations were designed as described by Liu and Naismith (2008) and are listed in Table S2 (see Supporting Information). High-fidelity Phusion DNA polymerase was used in polymerase chain reaction (PCR) mutagenesis (Finnzymes, Espoo, Finland). PCR products were treated with *DpnI* (New England Biolabs, Ipswich, MA, USA) for 3 h at 37 °C before being transformed into competent DH5 α cells. Plasmids were purified using a GenElute plasmid miniprep kit (Sigma-Aldrich, Hamburg, Germany). The mutated sites were confirmed by sequencing.

Sequence analysis

Sequences obtained from the curated DPVweb database (latest update in August 2013) were aligned using CLUSTALX 2.0. Grouping was performed according to a phylogenetic tree constructed by neighbour-joining methods in MEGA 5.0 (Tamura *et al.*, 2011). 3D modelling of HCpro and its variants was carried out using the I-TASSER webserver (Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, USA), and structures were viewed using PyMol (Roy *et al.*, 2010). The last sequence was submitted for modelling on 21 July 2014. The predicted models were refined by REMO and FG-MD analyses. The predicted 3D structures were aligned using Tm-Align (Zhang and Skolnick, 2005).

Similarities between the predicted model and structures in the Protein Data Bank (PDB) (<http://www.rcsb.org/>) were shown by Tm-Align during the prediction (Zhang and Skolnick, 2005). Only the 10 closest structural analogues were listed in the prediction results. The models of the most similar proteins in the PDB and PVY HCpro were superimposed using Pymol.

Intrinsically disordered regions of proteins were predicted using the metaDISORDER webserver, which calculates the consensus of 13 primary

methods, including DisEMBL (Linding *et al.*, 2003a), DISOPRED2 (Ward *et al.*, 2004), DISpro (Cheng *et al.*, 2005), Globplot (Linding *et al.*, 2003b), iPDA (Su *et al.*, 2007), IUPred (Dosztanyi *et al.*, 2005), Pdisorder (<http://www.softberry.com/berry.phtml?topic=pdisorder&group=programs&subgroup=propt>), Poodle-S (Shimizu *et al.*, 2007), Poodle-L (Hirose *et al.*, 2007), PrDOS (Ishida and Kinoshita, 2007), Spritz (Vullo *et al.*, 2006), DisPSSMP (Su *et al.*, 2006) and RONN (Yang *et al.*, 2005). For a more detailed description of the procedures, see Kozłowski and Bujnicki (2012). All residues were considered to be disordered when the disorder probability was greater than 0.5. The presence of a transmembrane helix was predicted in full-length HCpro amino acid sequences using ΔG analysis (<http://dgpred.cbr.su.se/>; Hessa *et al.*, 2007).

Virus inoculum

Plasmids containing PVY⁶⁰⁵ or other virus constructs were inoculated biolistically onto fully expanded leaves of 6-week-old tobacco plants using a HandyGun, as described by Sikorskaite *et al.* (2010). PVY⁰UK was inoculated mechanically to tobacco plants using sap extracted from infected leaves of potato 'Pito.' The upper non-inoculated leaves were observed for mosaic and veinal necrosis symptoms at 14 and 21 dpi, and tested for viruses by DAS-ELISA using non-strain-specific polyclonal anti-PVY CP (Bioreba, Reinach, Switzerland) at 11 dpi, as described by Rajamäki *et al.* (1998) and Tian and Valkonen (2013). Leaf samples were weighed, and 1 g of tissue was ground in 9 mL of ELISA sample buffer; two 100- μ L aliquots of sap were transferred to two wells of a microtitre plate (Greiner Labortechnik, Frickenhausen, Germany). Known amounts of purified virions of PVY⁰UK were included for comparison to estimate virus titres. The absorbance at 405 nm (A_{405}) was recorded using a microtitre plate reader (Benchmark; Bio-Rad, Hercules, CA, USA) and was regarded as positive if it was two times higher ($\pm$ standard deviation) than that of healthy control plants (Fenlon and Sopp, 1991). Systemically infected tobacco leaves tested by DAS-ELISA were stored at –80 °C, and the same patch of leaves was used for repeated potato plant inoculation experiments. These tobacco leaves were also tested by reverse-transcription PCR and direct sequencing of PCR products to verify that the introduced mutations had not reverted, and that no additional mutations had occurred in the 5'-proximal region of the viral genome during propagation in tobacco.

Testing of indicator potato cultivars for response to PVY

PVY⁰UK, PVY⁶⁰⁵ and all chimeras were tested on potato cvs. Pentland Crown, Pentland Ivory and King Edward in at least two independent experiments. Inocula were prepared by grinding PVY-infected tobacco leaf tissue at 1 g/10 mL sterile water in a chilled mortar with a pestle. Sap was rubbed onto potato leaves dusted with abrasive (silicon carbide; Carborundum, 400 mesh). The three first fully expanded leaves of 4-week-old potato plants were inoculated mechanically and marked by punching a hole in the leaf tip with the sterile tip of a micropipette. The development of local lesions on the inoculated leaves was monitored up to 21 dpi. One inoculated leaf of each plant was sampled at 12 dpi and tested for PVY by DAS-ELISA.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Comparison of three-dimensional (3D) models of the helper component proteinase (HCpro) in *Potato virus Y*^N (PVY^N), PVY^C and PVY^O strains and two additional chimeras.

Fig. S2 Comparison of the three-dimensional (3D) structural models of the helper component proteinase (HCpro) and β -catenin predicted by I-TASSER.

Table S1 Disorder tendency in the helper component proteinase (HCpro) from *Potato virus Y* (PVY) strains predicted by metaDISORDER (values ≥ 0.5 are shown).

Table S2 Primers used in this study.